THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 266, No. 15, Issue of May 25, pp. 9598-9604, 1991
Printed in U.S.A.

Expression, Isolation, and Characterization of an Active Site (Serine 528→Alanine) Mutant of Recombinant Bovine Prothrombin*

(Received for publication, December 14, 1990)

Gang Pei, Keith Baker‡, S. Michelle Emfinger‡, Dana M. Fowlkes‡, and Barry R. Lentz§

From the Department of Biochemistry and Biophysics and ‡Department of Pathology, University of North Carolina, Chapel Hill, North Carolina 27599-7260

An active site mutant bovine prothrombin cDNA (Ser^{528}→Ala) has been constructed, subcloned, and expressed in Chinese hamster ovary cells. The recombinant mutant prothrombin, expressed at the level of 1.5–2.0 μg/ml of cell medium, was fully carboxylated (9.9 ± 0.4 mol of γ-carboxyglutamic acid/mol of prothrombin). The mutant prothrombin could be activated to thrombin by Taipan snake venom and activated to meizothrombin by ecarin in a manner comparable to native bovine prothrombin or recombinant wild-type bovine prothrombin. The mutant meizothrombin thus formed was stable and did not autolyze. The initial rate of cleavage of mutant prothrombin catalyzed by the full prothrombinase was only 28% of the rate of cleavage of native prothrombin, while recombinant wild-type prothrombin was cleaved at the same rate as the native molecule. The mutant thrombin, obtained from the mutant prothrombin *in situ* by prothrombinase or Taipan snake venom activation, showed no enzymatic activity toward either fibrinogen or a synthetic chromogenic substrate, D-phenylalanyl-L-pipecolyl-L-arginine-*p*-nitroanilide dihydrochloride (S2238). The mutant thrombin also bound dansylarginine-*N*-(3-ethyl-1,5-pentanediyl)amide, a specific fluorescent inhibitor of the thrombin active site, with a weaker binding affinity (k_d = 5.4 × 10^{-8} M) than did native thrombin (k_d = 1.7 × 10^{-8} M). These results indicate that the mutant recombinant prothrombin described here is a useful tool for the study of meizothrombin or thrombin without the complications arising from the proteolytic activities of these molecules. Study of the activation of this mutant has already revealed a functional link between the site of initial cleavage by the prothrombinase and the conformation at the nascent active site of prothrombin.

Prothrombin is the precursor of thrombin, a serine protease which plays a central role in the process of blood coagulation (Mann *et al.*, 1988). The activation of bovine prothrombin to thrombin requires cleavage of two peptide bonds, Arg^{323}-Ile^{324} and Arg^{274}-Thr^{275}. Cleavage at Arg^{323}-Ile^{324} results in exposure of the active site and formation of meizothrombin, and, if Arg^{274}-Thr^{275} has already been cleaved, thrombin (Mann *et al.*, 1981; Rosing and Tans, 1988). Like all other serine proteases, the thrombin active site contains the catalytic triad, composed of His^{366}, Asp^{420}, and Ser^{528}.

The activity of thrombin active site toward fibrinogen, factor V, factor VIII, factor XIII, and platelets makes it very difficult to measure the thrombin binding to those substrates without activation. The cleavage of Arg^{156}-Ser^{157} in bovine prothrombin and of both Arg^{155}-Ser^{156} and Arg^{286}-Thr^{287} in human prothrombin by either thrombin or the activation intermediate meizothrombin also creates an obstacle to the crystallization of prothrombin or to biophysical studies of meizothrombin. Meizothrombin undergoes autolysis even in the presence of potent active site inhibitors (Armstrong *et al.*, 1990; Doyle and Mann, 1990; Pei and Lentz, 1991). Therefore, it is desirable to have an active site mutant of prothrombin.

A *wild-type human* prothrombin cDNA has been expressed in Chinese hamster ovary cells (CHO),[1] and the expressed recombinant prothrombin has been shown to be equivalent to plasma-derived prothrombin in specific coagulant activity and in γ-carboxyglutamic acid content (Jorgensen *et al.*, 1987). A series of point mutations in the amino-terminal region of prothrombin were successively made to identify the amino acid residues in the γ-carboxylation recognition site (Huber *et al.*, 1990). In the study reported here, a *wild-type bovine* prothrombin cDNA and an *active site mutant (Ser^{528}→Ala) bovine* prothrombin cDNA have been constructed and expressed in CHO cells. The mutant recombinant prothrombin obtained was fully γ-carboxylated, and was activated at similar rates by ecarin and Taipan snake venom and at a slower rate by the full prothrombinase as compared to native prothrombin or wild-type recombinant prothrombin. The mutant thrombin had no activity toward fibrinogen or toward a synthetic chromogenic substrate (S2238) and displayed a moderately decreased binding affinity toward dansylarginine-*N*-(3-ethyl-1,5-pentanediyl) amide (DAPA), a specific thrombin active site inhibitor.

EXPERIMENTAL PROCEDURES

Materials—Bovine brain phosphatidylserine (PS) and 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine (PC) were purchased from Avanti Biochemicals (Birmingham, AL). DAPA was synthesized and purified according to Mann *et al.* (1981). D-Phenylalanyl-L-pipecolyl-L-arginine-*p*-nitroanilide dihydrochloride (S2238) was from Helena Laboratories (Beaumont, TX). Ecarin from *Echis carinatus* snake venom and Taipan snake venom from *Oxyuranus scutellatus* were from Sigma. Morpholine was obtained from Aldrich and formaldehyde

* This work was supported by United States Public Health Service Grants HL26309 (Specialized Center for Research in Thrombosis and Hemostasis, to B. R. L.) and HL36197 and HL31012 (to D. M. F.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom reprint requests should be addressed.

[1] The abbreviations used are: CHO, Chinese hamster ovary; S2238, D-phenylalanyl-L-pipecolyl-L-arginine-*p*-nitroanilide dihydrochloride; DAPA, dansylarginine-*N*-(3-ethyl-1,5-pentanediyl)amide; PS, bovine brain phosphatidylserine; PC, 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine; ITS, insulin-transferrin-sodium selenite media supplement; SUV, small unilamellar vesicles; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Gla, γ-carboxyglutamic acid.

(37% aqueous solution) was from Mallinckrodt (St. Louis, MO). [^{14}C]Formaldehyde was purchased from Du Pont-New England Nuclear. Insulin-transferrin-sodium selenite media supplement (ITS) and NuSerum™ were from Collaborative Research (Bedford, MA). Cell growth medium Dulbecco's modified Eagle's medium/F-12 and calf serum were obtained from Linberger Cancer Research Center, University of North Carolina, Chapel Hill.

Isolation of bovine prothrombin and preparation of small unilamellar vesicles (SUV) of PS and PC (PS/PC, 25/75) were as described (Tendian and Lentz, 1990). Factor X and factor V were isolated from human plasma by procedures routinely used in our laboratory (Cutsforth *et al.*, 1989). Factor X was activated with a purified fraction of Russel's viper venom factor X-activating protein which had been covalently linked to agarose beads (Jesty and Nemerson, 1976). Factor V was activated by 1.25 ng/ml factor V-activating protein isolated from Russell's viper venom (Kisiel and Canfield, 1981) *in situ*. Thrombin was generated and purified according to the procedure of Church and Whinna (1986) and was coupled to agarose beads (Affi-Gel 10, Bio-Rad) following the instructions provided by Bio-Rad. Rabbit antisera were raised against purified bovine thrombin using incomplete Freund's adjuvant. Protein concentrations were determined by absorbance measurements using extinction coefficients at 280 nm of 1.44 ml mg^{-1} cm^{-1} for prothrombin and 1.05 ml mg^{-1} cm^{-1} for prothrombin fragment 1 (Mann, 1976).

Construction of Expression Plasmids, Wild-type and Mutant Bovine Prothrombin cDNA Clones—The expression vector used for these experiments was a derivative of a shuttle vector, pDx, from Dr. K. Berkner, ZymoGenetics (Seattle, WA). pDx was modified by the insertion of an assembled pair of oligonucleotides (aattcactcgagagcggccgc and aattgcggccgctctcgagtg) into the unique *Eco*RI site. This placed a unique *Not*I site immediately downstream of the unique *Eco*RI site. The resulting plasmid was called p272 (details not shown). New subcloning vectors were also constructed. p287 was derived from pBS(−) (Stratagene, San Diego) by cloning a small oligonucleotide fragment (agcttccatggtcgcgactcgagctgca and gctcgactcgcgaccatgga) encoding a *Nco*I restriction site between the *Hin*dIII and *Pst*I sites. p431 was constructed by inserting an oligonucleotide fragment (agcttgcggccgcctgcaggatatcccatggtggccag amd aattctggccaccatgggatatcctgcaggcggccgca) encoding *Not*I, *Pst*I, and *Nco*I sites between the *Hin*dIII and EcoRI sites of p287.

Two plasmids, pBII-3 and pBII-111, containing amino- and carboxyl-terminal portions of bovine prothrombin were obtained from Ross MacGillivray, University of British Columbia (MacGillivray *et al.*, 1980). The prothrombin-coding sequence containing the initiating methionine codon is located within a unique *Nco*I restriction enzyme recognition site in pBII-111. To obtain a full-length bovine prothrombin cDNA, we subcloned the 450-base pair *Pst*I-*Bam*HI fragment from pBII-3 and the 1500-base pair *Nco*I-*Bam*HI fragment into p287 (Fig. 1*A*). The resulting clone was called p429. The *Nco*I-*Pst*I fragment of p429, containing the complete prothrombin cDNA was subcloned into p431 to form p433. Hence, p433 contains a unique *Not*I site at the 3′ end of the prothrombin cDNA (Fig. 1*A*). The prothrombin expression vector, p437 (Fig. 1*C*), was constructed by subcloning the *Eco*RI-*Not*I fragment of p433 into the shuttle vector p272.

The 500-base pair *Bam*HI-*Hin*dIII fragment from p433 encoding the carboxyl-terminal region of prothrombin was subcloned into pUC-8, resulting in a clone called p479 (Fig. 1*B*). To mutate the active site serine (Ser^{528}) to alanine, we replace the *Bam*HI-*Ban*II fragment of p479 with a fragment assembled from four synthetic oligonucleotides to yield clone p481. The sequences of these oligonucleotides are gatccgcatcaccgacaacatgttctgtgccggttacaagcctggtgaaggc, aaacgaggggac gcttgtgagggcgacgcagggggacccttcgtcatgaagagcc, cttcatgacgaagggtccccc tgcgtcgccctcacaagcgtcccctc, gtttgccttcaccaggcttgtaaccggcacagaacatgtt gtcggtgatgcg. The *Bam*HI-*Hin*dIII fragment of p481 was subcloned into p433, resulting in p485 (see Fig. 1*B*). The region of Ser^{528}→Ala was sequenced by the dideoxynucleotide method (Sanger *et al.*, 1977) to confirm the presence of the Ser^{528}→Ala mutation and the lack of other undesirable mutations. The *Eco*RI-NotI fragment of p485 was subcloned into p272. The result is a shuttle expression vector, p487 (Fig. 1*C*) that has the potential to express Ser^{528}→Ala prothrombin.

Expression of Mutant and Wild-type Prothrombin in CHO Cells—Calcium phosphate cotransformation of CHO was carried out using a 1:10 ratio of p437 or p487 to pRSVneo according to a standard protocol (Graham and van der Eb, 1973). Cells were grown in Dulbecco's modified Eagle's medium/F-12 with 10% calf serum and 500 μg/ml G418, a neomycin analog (GIBCO Bethesda Research Laboratory). Individual clones of CHO cells resistant to G418 were tested for production of prothrombin. Nearly confluent cells were washed twice in Dulbecco's modified Eagle's medium/F-12/ITS and 10 μg/ml vitamin K. Two ml of media was left on each plate for 24 h. The media from the CHO cells transformed with wild-type prothrombin expression vector were tested for their ability to hydrolyze Spectrozyme TH as recommended by the supplier (American Diagnostica, NY) upon activation with ecarin. Six colonies produced significant levels of prothrombin. One of these has been used for the studies presented below. The media from CHO clones producing the mutant form of prothrombin were assayed for production of prothrombin using Western blot analysis with the rabbit antisera. One clone that produced detectable levels of mutant prothrombin has been used for the studies. Stocks of cells were maintained in Dulbecco's modified Eagle's medium/F-12 with 5.0% NuSerum, 5.0% calf serum, and 500 μg/ml G418.

To scale up cell production, one confluent 100-mm plate was transferred to one roller bottle (Costar, 900 cm^2) in a total volume of 250 ml. In about 4 days, the cells were confluent. Sterile microcarrier beads (0.8 g, Pharmacia LKB Biotechnology, Inc., Cytodex 2), diluted in 50 ml of growth medium, were added to each bottle. After 3 days, when the beads were covered with cells, they were washed once briefly with serum-free medium and once again overnight. All medium was removed, and each bottle was fed with 250 ml of serum-free medium. About 50 ml of conditioned medium was harvested daily, with 50 ml of fresh medium added, for 3 weeks. A final concentration of 10 mM benzamidine and 0.15 mM phenylmethylsulfonyl fluoride was added into the medium collected.

Isolation of Recombinant Prothrombins—Briefly, 3.7 ml of 0.4 M sodium citrate and 0.1 ml of 1.0 M benzamidine were added to 100 ml of the cell-free medium containing recombinant prothrombin. Prothrombin was precipitated with 1/10th volume of 1 M barium chloride, and the precipitate was washed three times with 0.1 M barium chloride and 1 mM benzamidine. The precipitate was resuspended in 40% saturated ammonium sulfate and 0.2 M EDTA and subjected to centrifugation. The supernatant was then dialyzed against 20 mM Tris, 1 mM benzamidine, pH 7.4, overnight and loaded onto a FPLC Mono Q ion-exchange column (HR 5/5, Pharmacia LKB Biotechnology, Inc.). The column was washed with 20 mM Tris, pH 7.4, buffer, and the protein was eluted with a linear gradient from 0 to 0.7 M NaCl in the same buffer at 1.5 ml/min. Protein absorbance was monitored at 280 nm. The identity of proteins in different fractions was determined by sodium dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), using prestained SDS-PAGE standards (Bio-Rad) and purified native prothrombin as the standard. The fractions containing pure prothrombin were combined and stored at −70 °C for the following studies.

γ-Carboxyglutamic Acid Content of Recombinant Prothrombins — Prothrombin fragment 1 was generated by cleavage of prothrombin with thrombin coupled to agarose beads. The resulting fragment 1 and prethrombin 1 were separated on a FPLC Mono Q column with a linear gradient of NaCl (0–0.7 M) in 20 mM Tris, pH 7.4, buffer. Taking native prothrombin fragment 1 as the standard, the relative γ-carboxyglutamic acid (Gla) content of fragment 1 of recombinant prothrombins was determined according to the Gla-specific formaldehyde-morpholine Mannich base fragmentation method (Wright *et al.*, 1986). This method converts the 10 Gla residues of prothrombin fragment 1 into 10 γ-methyleneglutamyl residues and can quantitatively label the Gla residues when [^{14}-C]formaldehyde is used. Protein concentration of the modified fragment F1 was determined by absorbance at 280 nm and ^{14}C radioactivity of the modified fragment F1 was counted on a LKB 1211 Rackbeta liquid scintillation counter.

Prothrombin Activation by Prothrombinase and Enzymatic Activity of the Activation Products—Mutant recombinant prothrombin, wild-type recombinant prothrombin, and native prothrombin (all at 1.2 μM) were activated with 0.5 nM factor X_a, 5 nM factor V_a, 10 μM PS/PC (25/75) SUV and 5 mM $CaCl_2$ in 20 mM Tris, 150 mM NaCl, pH 7.4, at 22 °C. At intervals of 0, 5, and 10 min, aliquots were withdrawn and subjected to SDS-PAGE (10% polyacrylamide) under nonreducing and reducing (3% 2-mercaptoethanol) conditions. Gels were stained with Coomassie Blue R-250 (Sigma), and the protein bands were quantitated by densitometry (Molecular Dynamics, Sunnyvale, CA) to estimate the initial rate of prothrombin activation.

Activation was also carried out at 37 °C and thereby taken to completion in 45 min. The product was thrombin in every case as shown by SDS-PAGE. An aliquot of each incubation was taken to measure the activity toward synthetic chromogenic substrate S2238 and fibrinogen (Sigma). The measurements were performed simultaneously on a 96-well plate with a Microplate Reader (model 3550, Bio-Rad) with absorbance read every 30 s at 405 nm. For the synthetic

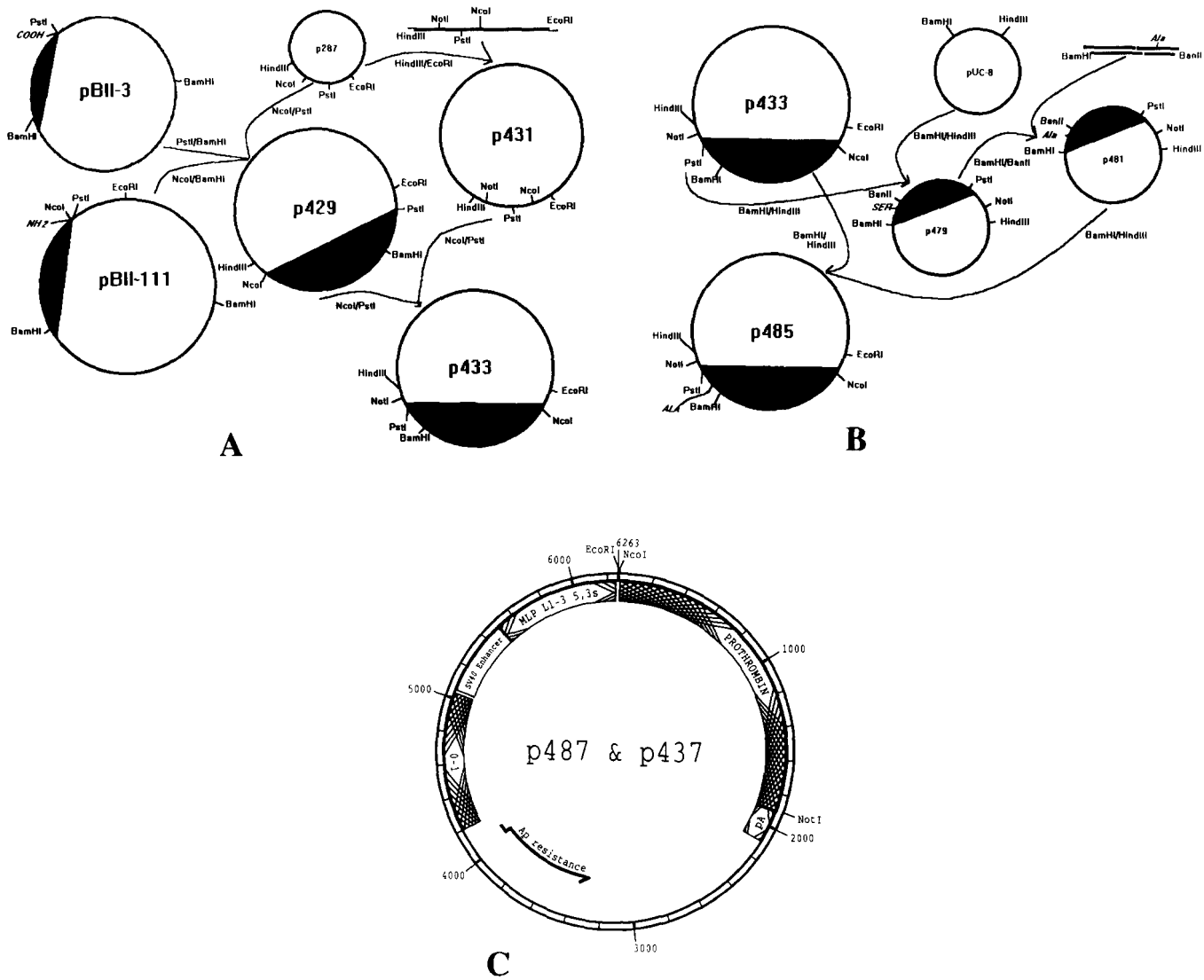

FIG. 1. **Schematic diagram of construction of the expression vector for recombinant prothrombins.** The expression vector for the wild type (p437) was made by subcloning the *Eco*RI-*Not*I fragment of p433 (*A*) into p272. The mutant prothrombin (Ser^{528}→Ala) expression vector (p487) was made by subcloning the *Eco*RI-*Not*I fragment of p485 (*B*) into p272 (see "Experimental Procedures" for details). Resulting vectors are shown in *C*.

chromogenic substrate assay, 5-μl aliquots (equal to 0.45 μg of prothrombin), 60 μl of 1 mM S2238, and 120 μl of 20 mM Tris, 150 mM NaCl, pH 7.4 buffer were used. For the fibrinogen clotting assay, 2-μl aliquots (equivalent to 0.18 μg of prothrombin), 140 μl of 5 mg/ml fibrinogen, and 50 μl of 20 mM Tris, 150 mM NaCl, pH 7.4 buffer were used, and turbidity was recorded at the same wavelength.

Prothrombin Activation by Ecarin and Taipan Venom—Recombinant prothrombins were also activated with ecarin from *E. carinatus* venom, which cleaves the Arg^{323}-Ile^{324} bond of bovine prothrombin (Rosing and Tans, 1988). Ecarin (12.5 μg/ml final concentration) was added to a final concentration of 90 μg/ml of native or recombinant prothrombin in 20 mM Tris, 150 mM NaCl, 6 mM $CaCl_2$, pH 7.4, buffer at 37 °C to start the activation. At intervals of 0, 30, 90 min, 20-μl aliquots were withdrawn and subjected to SDS-PAGE under both nonreducing and reducing conditions. Gels were stained with Coomassie Blue R-250.

The DAPA fluorescence assay (Mann *et al.*, 1981; Nesheim and Mann, 1983; Krishnaswamy *et al.*, 1986) was also applied to compare the activation of mutant and native prothrombin by ecarin and Taipan venom. Taipan snake venom from *O. scutellatus* is known to activate prothrombin to thrombin in the absence of factor V_a at a rate that is enhanced 20–30 times by phospholipid vesicles (Owen and Jackson, 1973). Using an SLM 48000® fluorometer with excitation wavelength at 280 nm and emission wavelengths at 515–550 nm with a 515-nm cut-off filter, 125 nM mutant or native prothrombin, 5 mM $CaCl_2$, and 500 nM DAPA in 20 mM Tris, 150 mM NaCl, pH 7.4, buffer were incubated at 37 °C for 3 min before adding 100 μg/ml ecarin or 10 μg/ml Taipan venom (final concentrations) to start the reaction. The Taipan venom activation mixture also contained 10 μM PS/PC (25/75) SUV. The fluorescence intensity of DAPA was greatly enhanced when bound to the active site of newly formed meizothrombin or thrombin, and this allowing us to record continuously the time course of prothrombin activation. The fluorescence emission spectra of both reaction mixtures were also determined from 450 to 620 nm (4-nm bandpass) with an excitation wavelength of 280 nm and a 450-nm cut-off filter in the emission beam.

DAPA Fluorescence Emission Intensity Titration with Mutant or Native Thrombin—1.6 μM mutant or native prothrombin was incubated with 50 μg/ml Taipan venom, 10 mM PS/PC (25/75) SUV, and 5 mM $CaCl_2$ in 20 mM Tris, 150 mM NaCl, pH 7.4, buffer at 22 °C overnight. Prothrombin was completely activated to thrombin as judged by SDS-PAGE.

The activation mixtures were then used to titrate 150 nM DAPA in 20 mM Tris, 150 mM NaCl, pH 7.4, at 22 °C by measuring DAPA fluorescence emission intensity using λ_{ex} = 280 nm and λ_{em} = 515–550 nm with a 515 nm cut-off filter. The data were analyzed by the equation (Nesheim *et al.*, 1979; Hibbard *et al.*, 1982)

$$\frac{1}{f[L]_0} = \frac{n[P]_0}{b[L]_0 K_d} - \frac{1}{K_d} \quad (1)$$

in which $[P]_0$ and $[L]_0$ are the nominal concentrations of protein and ligand, respectively; f and b are the fraction of total ligand concentration free and bound, respectively; n is the number of independent, noninteracting ligand-binding sites/protein molecule, and K_d is the dissociation constant.

RESULTS

FPLC and SDS-PAGE Behaviors of Native and Recombinant Prothrombins —Recombinant prothrombins isolated after barium precipitation and ion-exchange FPLC were nearly homogeneous as judged by SDS-PAGE (data not shown). Recombinant prothrombins migrated the same as native prothrombin on SDS-PAGE under both nonreducing and reducing conditions (Fig. 2). When eluted with a NaCl gradient from a Mono Q column, wild-type recombinant prothrombin was eluted at 21.14 min and mutant recombinant prothrombin (Ser^{528}→Ala) was eluted at 21.01 min as compared to 21.26 min for native bovine prothrombin. After the FPLC purification, the yields of wild-type and mutant prothrombin were 0.6–0.8 and 1.5–2.0 μg/ml of cell medium, respectively.

Recombinant Prothrombins Possess Normal Gla Content— The relative γ-carboxyglutamic acid (Gla) contents of the fragment 1 peptides isolated from recombinant and native prothrombins were determined by the Gla-specific [^{14}C]formaldehyde-morpholine method (Wright *et al.*, 1986). This method specifically labels Gla residues with [^{14}C]formaldehyde. The results are presented in Table I, where the specific ^{14}C radioactivity of each modified fragment 1 was obtained using the molecular weight of 22,000 (Mann, 1976). Using the ratio of the specific radioactivity of recombinant prothrombin fragment 1 to that of the native molecule and a value of 10 Gla residues/molecule (Nelsestuen *et al.*, 1974) for native prothrombin fragment 1, the relative values of Gla content of recombinant prothrombin fragment 1 were calculated (Jorgensen *et al.*, 1987). Mutant recombinant prothrombin contained 9.9 ± 0.5 mol of Gla residues/mol of fragment 1, and wild-type recombinant prothrombin contained 9.4 ± 0.4 mol of Gla residues/mol of fragment 1.

TABLE I

γ-Carboxyglutamic acid content of fragment 1 of recombinant prothrombins

	Specific activity	γ-Carboxyglutamic acid content
	dpm/pmol	*mol/mol*
Native prothrombin F1	34.6 ± 1.5	10.0[a]
Mutant prothrombin F1	34.3 ± 1.6	9.9 ± 0.5
Wild-type prothrombin F1	32.5 ± 1.3	9.4 ± 0.4

[a] 10 Gla residues/native prothrombin fragment 1 was taken as the standard to obtain relative values for the recombinant molecules.

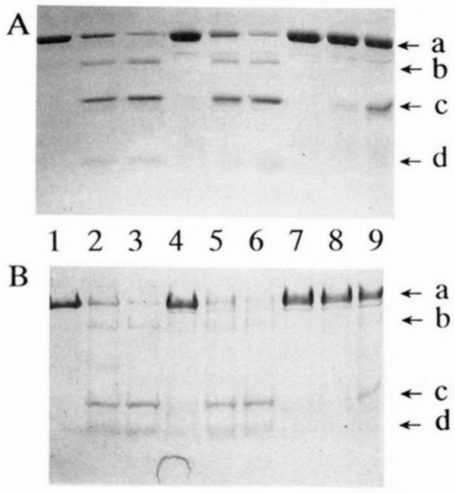

FIG. 2. **SDS-PAGE analysis of recombinant prothrombin activation by the full prothrombinase complex.** Recombinant and native prothrombins (1.2 μM) were activated to thrombin with 0.5 nM factor X_a, 5 nM factor V_a, 10 μM PS/PC (25/75) SUV, and 5 mM $CaCl_2$ in 20 mM Tris, 150 mM NaCl, pH 7.4, at 22 °C. At intervals of 0, 5, and 10 min, aliquots were withdrawn and subjected to SDS-PAGE under nonreducing (*A*) and reducing (3% of 2-mercaptoethanol, *B*) conditions. *Lanes 1–3* are native bovine prothrombin, *lanes 4–6* are wild-type recombinant prothrombin, and *lanes 7–9* are mutant recombinant prothrombin (Ser^{528}→Ala). The indicated bands in *A* are *a*, prothrombin; *b*, meizothrombin des fragment 1 or prethrombin 1; *c*, thrombin; and *d*, fragment 1. The bands in *B* are *a*, prothrombin; *b*, prethrombin 1; *c*, fragment 1·2 and/or thrombin B chain; *d*, fragment 1.

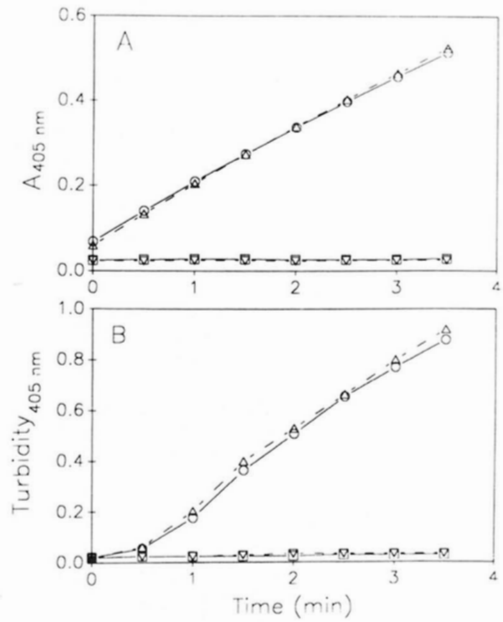

FIG. 3. **Enzymatic activities of prothrombin activation products toward S2238 and fibrinogen.** 1.2 μM native or recombinant prothrombins were activated with 0.5 nM factor X_a, 5 nM factor V_a, 10 μM PS/PC (25/75) SUV, and 5 mM $CaCl_2$ in 20 mM Tris, 150 mM NaCl, pH 7.4, at 37 °C for 45 min. An aliquot of each incubation was taken to test the activity toward synthetic chromogenic substrate S2238 (*A*) and fibrinogen (*B*). Data are shown in both figures for native bovine prothrombin (*circles*), wild-type recombinant prothrombin (*triangles*), mutant recombinant prothrombin (*squares*), and the control incubation without prothrombin (*inverted triangles*).

Activation of Recombinant Prothrombins by Prothrombinase—As shown in Fig. 2, activation of wild-type recombinant prothrombin by the full prothrombinase complex was similar both in rate and extent of activation to the activation of native prothrombin. By contrast, the activation of the mutant prothrombin (Ser^{528}→Ala) was much slower than that of the native molecule under the same conditions. The initial rate of recombinant prothrombin activation relative to that of native prothrombin, as quantitated with the scanning densitometer, was 97% for wild-type prothrombin and 28% for mutant prothrombin.

Mutant Thrombin Showed No Activity Toward S2238 and Fibrinogen—Thrombin derived either from wild-type prothrombin or mutant prothrombin by the prothrombinase activation was tested for its activities toward synthetic chromogenic substrate S2238 and fibrinogen and compared to native bovine thrombin (Fig. 3). As expected, wild-type thrombin had the same enzymatic activities as native thrombin, while the mutant thrombin (Ser^{528}→Ala) displayed no

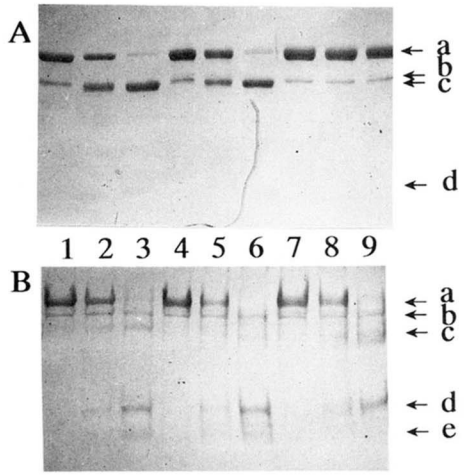

FIG. 4. **SDS-PAGE analysis of recombinant prothrombin activation by ecarin.** 12.5 μg/ml ecarin (final concentration) was added to 90 μg/ml of native or recombinant prothrombins in 20 mM Tris, 150 mM NaCl, 6 mM $CaCl_2$, pH 7.4, buffer at 37 °C to start the activation. At 0, 30, and 90 min, 20 μl of aliquot were withdrawn and subjected to SDS-PAGE under both nonreducing (*A*) and reducing conditions (3% of 2-mercaptoethanol, *B*). *Lanes 1–3* are native bovine prothrombin, *lanes 4–6* are wild-type recombinant prothrombin, and *lanes 7–9* are mutant recombinant prothrombin (Ser^{528}→Ala). The indicated bands in *A* are *a*, prothrombin; *b*, ecarin; *c*, meizothrombin des fragment 1; and *d*, fragment 1. The bands in *B* are *a*, prothrombin; *b*, ecarin; *c*, fragment 1·2-thrombin A chain; *d*, thrombin B chain; and *e*, fragment 1.

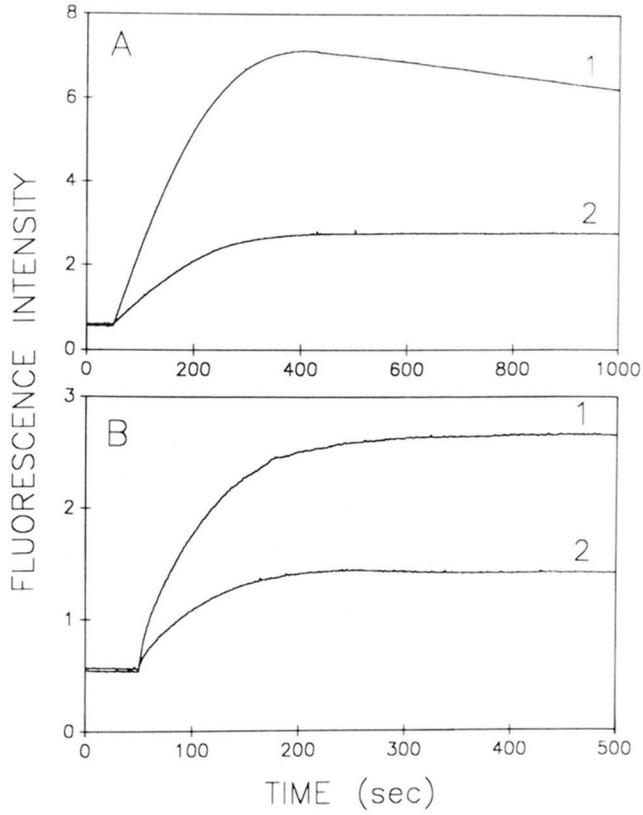

FIG. 5. **Mutant recombinant prothrombin activation by ecarin or Taipan venom as continuously monitored with DAPA fluorescence emission intensity.** The excitation wavelength was 280 nm and emission wavelengths were from 515 to 550 nm with a 515-nm cut-off filter. Reaction mixtures containing 125 nM native (*line 1*) or mutant prothrombin (*line 2*), 500 nM DAPA, and 5 mM $CaCl_2$ in 20 mM Tris, 150 mM NaCl, pH 7.4, at 37 °C were initiated by the addition of 120 μg/ml ecarin (*A*) or 10 μg/ml Taipan venom (*B*). The reaction mixture (*B*) also contained 10 μM PS/PC (25/75) SUV.

activity toward these substrates.

The reaction mixtures of the recombinant prothrombins activated by Taipan snake venom, in which thrombin was the activation product as judged by SDS-PAGE, were also tested. The same results (data not shown) were obtained as were obtained with the prothrombinase-derived thrombin.

SDS-PAGE Analysis of Recombinant Prothrombin Activation by Ecarin—When activated with ecarin, wild-type recombinant prothrombin behaved the same as native prothrombin, as analyzed with SDS-PAGE (Fig. 4). Activation of the wild-type prothrombin was almost completed at 90 min, and most prothrombin was converted to meizothrombin des fragment 1, which is the product of bovine meizothrombin autolysis (Rosing and Tans, 1988). Activation of mutant prothrombin was also completed by 90 min as judged by the reducing gel (Fig. 4*B*), but the product was meizothrombin instead of meizothrombin des fragment 1, showing mutant meizothrombin was not subject to autolysis as were the native and wild-type molecules.

DAPA Assay of Mutant Prothrombin Activation by Ecarin or Taipan Venom—When monitored with the DAPA fluorescence assay, the emission intensity of the mutant prothrombin reaction mixture activated by ecarin (*line 2*, Fig. 5*A*) reached a plateau after 300 s. The native prothrombin reaction mixture (*line 1*, Fig. 5*A*) also reached a maximum at about 300 s and subsequently declined. The later behavior is characteristic of formation of meizothrombin followed by its subsequent autolysis (Hibbard *et al.*, 1982; Doyle and Mann, 1990), as shown in the SDS-PAGE results in Fig. 4. Also consistent with Fig. 4, the initially formed mutant meizothrombin was stable (*line 2*, Fig. 5*A*).

The most interesting aspect of the data shown in Fig. 5 is that the fluorescence intensity of the mutant prothrombin activation mixture was only 33% of that of native molecule at the maximum when activated by ecarin. In addition, the fluorescence intensity after activation of mutant prothrombin to thrombin with Taipan venom was only 40% of the fluorescence intensity observed for the native thrombin-DAPA complex. This could be due either to reduced binding of DAPA or to a reduced quantum yield of the DAPA-mutant active site complex as compared to the DAPA-native active site complex. The DAPA fluorescence emission spectrum of the mutant prothrombin reaction mixture activated by ecarin was similar ($\lambda_{max} \approx 525$ nm) to that of the native activation mixture, measured at about 300 s after initiation of the activation (data not shown). The DAPA fluorescence emission spectrum of the mutant prothrombin reaction mixture activated by Taipan snake venom was also similar ($\lambda_{max} \approx 540$ nm) to that of the native activation mixture (data not shown).

Mutated Thrombin Active Site Showed a Weaker DAPA Binding Affinity—As titrated with the same amount of DAPA under the same conditions, the fluorescence intensity of the complex of DAPA and mutant thrombin was 74% of that of the complex of DAPA and native thrombin when both titrations reached saturation (data not shown). Analysis of the titration data using Equation 1 gave a stoichiometry $n = 1.22$ mol of DAPA/mol of mutant thrombin and a dissociation constant $K_d = 5.4 \times 10^{-8}$ M (Fig. 6*B*) as contrasted to $n = 1.02$ mol of DAPA/mol of native thrombin and $K_d = 1.7 \times 10^{-8}$ M (Fig. 6*A*). By comparison, $n = 1.20 \pm 0.14$ mol of DAPA/mol of bovine thrombin and $K_d = (1.9 \pm 0.6) \times 10^{-8}$ M were reported by Hibbard *et al.* (1982).

DISCUSSION

The catalytic triad residues in thrombin, as for other members of the serine protease family, are identified as His^{366},

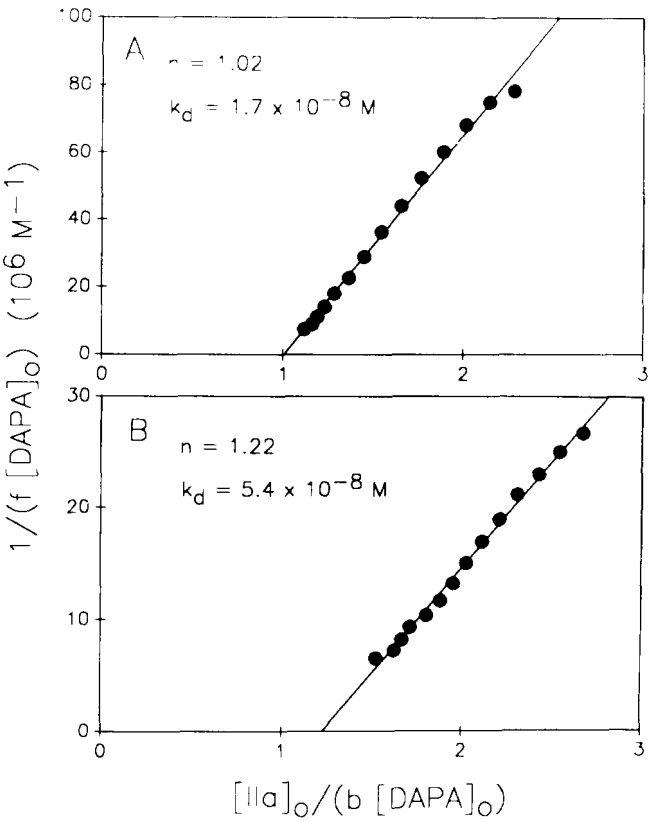

FIG. 6. **Analysis of DAPA titration with native thrombin (*A*) and mutant thrombin (*B*).** 1.6 μM native or mutant prothrombin was incubated with 50 μg/ml Taipan venom, 10 mM PS/PC (25/75) SUV, and 5 mM $CaCl_2$ in 20 mM Tris, 150 mM NaCl, pH 7.4, at 22 °C overnight. The reaction mixtures were then used to titrate 150 nM DAPA in 20 mM Tris, 150 mM NaCl, pH 7.4, at 22 °C by measuring DAPA fluorescence emission intensity with λ_{ex} = 280 nm. Data were analyzed according to Equation 1 under "Experimental Procedures" (Nesheim *et al.*, 1979; Hibbard *et al.*, 1982).

Asp^{420}, and Ser^{528} (Mann *et al.*, 1981). Because of the essential function of thrombin in blood coagulation, no natural prothrombin mutated in any of these 3 catalytic residues is likely to be found. In the current study, a mutant bovine prothrombin (Ser^{528}→Ala) cDNA has been constructed, subcloned, and expressed in CHO. The expressed mutant recombinant prothrombin, as compared to native prothrombin, is fully γ-carboxylated in CHO cells in the presence of vitamin K at a yield of 1.5–2.0 μg/ml. The mutant prothrombin (Ser^{528}→Ala), with the same mobility as native prothrombin on an anion exchange FPLC column and on SDS-PAGE, can be activated to meizothrombin and thrombin as are native and wild-type expressed prothrombin. The mutant meizothrombin obtained by ecarin activation is very stable and does not undergo autolysis even in the absence of thrombin active site inhibitors. Thus, it provides a useful tool to examine the role of meizothrombin as an important intermediate in prothrombin activation by the full prothrombinase. The availability of a stable form of meizothrombin will allow determination of the secondary or tertiary structure of meizothrombin as well as of its interactions with other coagulation factors and with the phospholipid surface. The mutant thrombin, as expected, does not cleave fibrinogen and does not hydrolyze thrombin-specific chromogenic substrates. This inactive mutant thrombin is suitable for studying the binding interactions of thrombin with its substrate and with inhibitors in the absence of catalysis.

The successful expression of the mutant prothrombin *in vitro* might also benefit crystallographic studies of prothrombin, thrombin, and even meizothrombin. So far, only the crystallographic structures of prothrombin fragment 1 and of thrombin inhibited with D-Phe-Pro-Arg chloromethylketone or hirudin have been reported (Tulinsky *et al.*, 1988; Skrzypczak-Jankun *et al.*, 1989; Bode *et al.*, 1989; Rydel *et al.*, 1990; Grütter *et al.*, 1990).

DAPA, a specific inhibitor of the thrombin active site (K_d = 1.7 × 10^{-8} M), does not bind to prothrombin or to prethrombin 1, in which both Arg^{274}-Thr^{275} and Arg^{323}-Ile^{324} remain intact, while it binds at a lower affinity (K_d = 5.9 × 10^{-7} M) to prethrombin 2, in which Arg^{323}-Ile^{324} is intact and the active site unexposed (Hibbard *et al.*, 1982). Although the replacement of Ser^{528} by Ala in the mutant thrombin active site reduces its binding affinity to DAPA about 3-fold (K_d = 5.4 × 10^{-8} M), the affinity is still 10 times higher than that of the DAPA-prethrombin 2 complex. In addition, the DAPA fluorescence emission spectrum of the mutant thrombin has the same spectral features as that of native thrombin. These observations suggest that the active site, after the Arg^{323}-Ile^{324} bond is cleaved, might be normally developed in the mutant thrombin, despite any disruption that might occur as a result of the Ser^{528}→Ala replacement. The weaker binding of the mutant thrombin to DAPA reveals that Ser^{528} of the catalytic triad may be directly involved in DAPA binding, as recently shown for hirudin binding (Rydel *et al.*, 1990).

It is worthwhile to point out that the initial rate of recombinant mutant prothrombin activation by the full prothrombinase is less than one-third the rate of native prothrombin activation, while the rate of activation of recombinant wild-type prothrombin was the same as for native prothrombin. By comparison, the activation rates of recombinant mutant prothrombin by ecarin (at Arg^{323}-Ile^{324}) and by Taipan snake venom (at both Arg^{274}-Thr^{275} and Arg^{323}-Ile^{324}) were the same as for native prothrombin and for recombinant wild-type prothrombin. This suggests that the mutation of Ser^{528} to Ala in the active site might alter the environment or structure around Arg^{323}-Ile^{324} in a way that could be sensed by the full prothrombinase, which activates prothrombin through meizothrombin as the intermediate (Rosing *et al.*, 1986; Krishnaswamy *et al.*, 1986). The prothrombin molecule has been shown to interact functionally with each component of the prothrombinase, factor X_a, factor V_a, Ca^{2+}, and the phospholipid surface (Mann *et al.*, 1988; Krishnaswamy 1990; Mann *et al.*, 1990). The difference in the kinetics of activation between mutant recombinant prothrombin and the native molecule might be derived, then, from altered molecular interactions between mutant prothrombin and some components of the prothrombinase. Alternatively, meizothrombin may function as an important catalytic species within the prothrombinase complex. If so, meizothrombin would be an ideal target for rational antithrombotic drug design. Use of mutant prothrombin and its activation products to investigate further the details of these interactions should lead to a better understanding of the structural basis of prothrombin activation by prothrombinase.

REFERENCES

Armstrong, S. A., Husten, E. J., Esmon, C. T., and Johnson, A. E. (1990) *J. Biol. Chem.* **265,** 6210–6218

Bode, W., Mayr, I., Baumann, U., Huber, R., Stone, S. R., and Hofsteenge, J. (1989) *EMBO J.* **8,** 3467–3475

Church, F. C., and Whinna, H. C. (1986) *Anal. Biochem.* **157,** 77–83

Cutsforth, G. A., Whitaker, R. N., Hermans, J., and Lentz, B. R. (1989) *Biochemistry* **28,** 7453–7461

Doyle, M. F., and Mann, K. G. (1990) *J. Biol. Chem.* **265,** 10693–10701

Graham, F. L., and van der Ed, A. J. (1973) *Virology* **52,** 456–461

Grütter, M. G., Priestle, J. P., Rahuel, J., Grossenbacher, H., Bode, W., Hofsteenge, J., and Stone, R. S. (1990) *EMBO J.* **9,** 2361–2365
Hibbard, L. S., Nesheim, M. E., and Mann, K. G. (1982) *Biochemistry* **21,** 2285–2292
Huber, P., Schmitz, T., Griffin, J., Jacobs, M., Walsh, C., Furie, B., and Furie, B. C. (1990) *J. Biol. Chem.* **265,** 12467–12473
Jesty, J., and Nemerson, Y. (1976) *Methods Enzymol.* **45,** 95–107
Jorgensen, M. J., Cantor, A. B., Furie, B. C., and Furie, B. (1987) *J. Biol. Chem.* **262,** 6729–6734
Kisiel, W., and Canfield, W. M. (1981) *Methods Enzymol.* **80,** 275–285
Krishnaswamy, S. (1990) *J. Biol. Chem.* **265,** 3708–3718
Krishnaswamy, S., Mann, K. G., and Nesheim, M. E. (1986) *J. Biol. Chem.* **261,** 8977–8984
MacGillivray, R. T. A., Friezner, D. S. J., Chandra, T., Woo, S. L. C., and Davie, E. W. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77,** 5153–5157
Mann, K. G. (1976) *Methods Enzymol.* **45,** 123–156
Mann, K. G., Elion, J., Butkowski, R. T., Downing, M., and Nesheim, M. E. (1981) *Methods Enzymol.* **80,** 286–302
Mann, K. G., Jenny, R. J., and Krishnaswamy, S. (1988) *Annu. Rev. Biochem.* **57,** 915–956
Mann, K. G., Nesheim, M. E., Church, W. R., Haley, P., and Krishnaswamy, S. (1990) *Blood* **76,** 1–16
Nelsestuen, G. L., Zytkovicz, T. H., and Howard, J. B. (1974) *J. Biol. Chem.* **249,** 6347–6350
Nesheim, M. E., and Mann, K. G. (1983) *J. Biol. Chem.* **258,** 5386–5391
Nesheim, M. E., Prendergast, F. G., and Mann, K. G. (1979) *Biochemistry* **18,** 996–1003
Owen, W. G., and Jackson, C. M. (1973) *Thromb. Res.* **3,** 705–714
Pei, G., and Lentz, B. R. (1991) *Blood Coag. Fibrinolysis,* in press
Rosing, J., and Tans, G. (1988) *Thromb. Haemostasis* **60,** 355–360
Rosing, J., Zwaal, R. F. A., and Tans, G. (1986) *J. Biol. Chem.* **261,** 4224–4228
Rydel, T. J., Ravichandran, K. G., Tulinsky, A., Bode, W., Huber, R., Roitsch, C., and Fenton, J. W., II (1990) *Science* **249,** 277–280
Sanger, F., Nicklen S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74,** 5463–5467
Skrzypczak-Jankun, E., Rydel, T. J., Tulinsky, A., Fenton, J. W., II, and Mann, K. G. (1989) *J. Mol. Biol.* **206,** 755–757
Tendian, S. W., and Lentz, B. R. (1990) *Biochemistry* **29,** 6720–6729
Tulinsky, A., Park, C. H., and Skrzypczak-Jankun, E. (1988) *J. Mol. Biol.* **202,** 885–901
Wright, S. F., Berkowitz, P., Deerfield, D. W., Byrd, P. A., Olson, D. L., Larson, R. S., Hinn, G. C., Koehler, K. A., Pedersen, L. G., and Hiskey, R. G. (1986) *J. Biol. Chem.* **261,** 10598–10605